POLY-(ADP) RIBOSE POLYMERASE ENZYME AND USES THEREOF

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[0001] CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application claims benefit under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 60/399,460, filed July 31, 2002, incorporated herein in its entirety.

[0003] STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0004] This invention was made with U.S. government support under grant number GM-27875 awarded by the National Institutes of Health. The U.S. government has certain rights in the invention.

[0005] BACKGROUND OF THE INVENTION

[0006] Field of the Invention

[0007] The present invention relates to the identification and cloning of a poly(ADP-ribose) polymerase (PARP) enzyme lacking catalytic activity and methods of modulating chromatin structure.

[0008] Related Art

[0009] Cells within multicellular eucaryotes as they develop build complex tissue-specific chromatin architectures to express certain genes and silence others (reviewed in Farkas et al. 2000. Gene 253:117-36). These intricately acquired chromatin domains must be preserved when chromosomal DNA is accessed for replication and repair, and when reprogramming is required it must be precisely targeted. In diverse eukaryotes, protein ADP-ribosylation plays important but imperfectly understood roles in apoptosis, gene transcription and in preserving chromatin during DNA repair (De Murcia, G., and Shall, S. 2000. From DNA damage and stress signalling to cell death. Poly (ADP-Ribosylation) Reactions. (New York: Oxford University Press; Ziegler et al. 2001. *Bioessays.* **23:** 543-548).

[0010] Poly(ADP-ribose) polymerase-1 (PARP1) is the major nuclear source of this activity in mice. The zinc fingers of the PARP1 protein specifically recognize DNA nicks and breaks and PARP activity is strongly increased upon binding to such sites. The bound, activated protein

transfers multiple ADP-ribose moieties from NAD onto local chromatin proteins such as histones, topoisomerases, polymerases and transcription factors (Poirier et al. 1982. *Proc. Natl. Acad. Sci. USA.* 79: 3423–3427; Menissier-de Murcia et al. 1997. *Proc. Natl. Acad. Sci. USA.* 94: 7303–7307). These modifications facilitate base excision repair by transiently dissociating target proteins from the chromosome to expose the lesioned area, by down regulating transcription of the affected genes, and by modulating the activity of checkpoint and stress regulatory proteins. The newly repaired region returns to a normal state after PARP downregulates its own activity by automodifying a specific domain and the chromatin proteins, freed of ADP-ribose groups by a specific glycosylase, reassemble. In contrast, if damage it too extensive, PARP is specifically inactivated by caspase cleavage as the cell commits to apoptosis (Kim *et al.* 2000).

[0011] A great deal of biochemical and cellular evidence supports the idea that PARP removes chromatin within damaged regions to facilitate DNA repair (de Murica, 1999). Moreover, mice mutant for *Parp1,* one of at least five murine genes encoding PARP-related proteins, though viable and fertile, are severely compromised in their ability to repair DNA lesions (Dantzer et al. 1998. *Nucleic Acids Res.* 26: 1891-1898). Mice with defective *PARP1* genes develop into fertile adults, hence a developmental role for PARP1 has yet to be established (Wang *et al.* 1995). However, four other mouse genes encode distinct ADP-ribosyl transferases with related catalytic domains (Amé *et al.* 1999. *J. Biol. Chem.* 274: 17860-17868; Kickhoefer et al. 1999. *J. Cell Biol.* 146: 917-928), including a telomere-associated form known as Tankyrase (Smith et al. 2001. *Science.* **282:** 1484-1487), so functional redundancy may have obscured such a role.

[0012] Data suggesting that PARP-mediated chromatin stripping is used in other contexts has been lacking. For example, *Parp1* knockout mice in addition to their damage susceptibility display dramatic immune defects, characterized by an inability to induce genes controlled by NF-κB transcription factors (Kameoka *et al.* 2000. *Biochem J*: 346:641-649). However, this defective immune response may be explained by the disruption of specific complexes that PARP forms with transcription factors such as YY1 (Oei *et al.* 1997. *Biochem. Biophys. Res. Commun.* 240:108–111), p53 (Mendoza-Alvarez *et al.* 2001), PAX6 (Plaza *et al.* 1999. *Oncogene.* 18:1041–1051), and NF-κB itself (Hassa *et al.* 1999. *Biol. Chem.* 380, 953–959) rather than by action at the chromatin level. Consequently, roles for PARP beyond its duties as a stress response regulator and transcriptional cofactor remain to be established.

[0013] The model eukaryote, *Drosophila melanogaster*, has the potential to support detailed genetic studies of PARP function in both physiology and development. Its genome contains a single gene, *Parp*, related to mammalian *Parp1* (Uchida *et al.* 1993. *Proc. Natl. Acad. Sci. USA.*

90: 3481-3485; Hanai *et al.* 1998. *J. Biol. Chem.* 273: 11881–11886), and one homologue of *tankyrase* (Adams *et al.* 2000. *Science* 287: 2185-2195). The protein specified by the major *Parp* transcript, PARP-I, includes all the conserved domains characteristic of mammalian PARP1 except a canonical caspase cleavage site. Parp-I transcripts are expressed in nearly mature ovarian follicles and throughout embryonic development, but were not detected in larvae (Hanai *et al.* 1998). Parp-II transcripts lacking the automodification domain are produced via differential splicing of a single exon (Kawamura *et al.* 1998. *Biochem Biophys Res. Commun.* 251: 35-40). However, genetic studies have been hindered because *Parp* is located deep within centromeric heterochromatin, and its exons are scattered among several contigs that remain unlinked to the euchromatic genome sequence (Adams *et al.* 2000).

[0014] *Drosophila* development has been extensively studied to determine how changes in chromatin structure contribute to specifying programs of tissue-specific and temporally regulated gene expression (reviewed in Farkas *et al.* 2000; Gerasimova *et al.* 2001. *Annu. Rev. Genet.* 35: 193-208). Zygotic transcription begins during the first 14 embryonic nuclear cycles concomitant with the establishment of heterochromatin and of nucleolus formation (Foe *et al.* B. 1983. *J. Cell Sci.* 61: 51-70). During subsequent embryonic and larval stages, chromatin domains are refined under the control of multi-protein remodeling complexes (reviewed by Cairns, B.R. 1998. *Trends Biochem. Sci.* 23: 20-25.; Jacobs *et al.* 1999. *Semin. Cell Dev. Biol.* 10: 227-235). The role of NAD-requiring enzymes in these processes is poorly known, but in addition, *Parp Drosophila* contain a gene structurally and functionally related to the NAD-dependent histone deacetylase encoded by the yeast *Sir2* locus (Barlow *et al.* 2001. *Exp. Cell. Res.* 265: 90-103; Rosenberg et al. 2002. *Cell.* 109: 447-458).

[0015] However, recent genetic studies in *Drosophila melanogaster* show that PARP plays a much more general role by organizing chromatin at multiple points throughout the life cycle (Tulin *et al.* 2002). Flies bearing mutations in the single Drosophila PARP gene display extensive changes in both the repression and activation of chromosome domains, and die during the transition between the 2nd and 3rd larval instar. Heterochromatin remains abnormally accessible to nuclease, and the transcription of certain repeated sequences such as the copia retrotransposon fails to be repressed. Nucleoli are defective, and at least some specific genes seem also to misfunction as *Parp* mutant larvae frequently arrest development during metamorphosis. Tulin *et al.* (2002) proposed that the genetic requirement for PARP resulted from its involvement in locally stripping and re-assembling chromatin under developmental control. However, it is difficult to rule out that these effects were secondary to disruption of transcriptional co-activation.

[0016] Drosophila chromatin normally undergoes many highly programmed changes during embryogenesis that could be targets of PARP action (reviewed in Farkas *et al.* 2000). These events continue during larval development through the action of chromatin remodeling complexes (reviewed in Simon et al. 2002. *Curr Opin Genet Dev.* 12: 210) and histone modifications (reviewed in Wolffe and Guschin, 2000). The larval polytene chromosomes reveal that dramatic programmed chromatin alterations continue within specific euchromatic regions that form puffs at the site of newly activated genes (reviewed by Ashburner and Berendes, 1978). Many developmental puffs are induced by the moulting hormone ecdysone and contain steroid hormone response genes or their targets (reviewed by Thummel. 2000. *Insect Biochem. Mol. Biol.* 32:113-120).

[0017] Others puffs are rapidly induced at the sites of stress response genes following heat shock (reviewed by Farkas *et al.* 2000). Despite their association with induced transcription, puffs are neither necessary nor sufficient for high level gene transcription (Meyerowitz *et al.* 1985), and their biological significance has remained unclear.

[0018] Thus, the effects of proteins on chromatin structure are varied and influence gene transcription and expression. There is a clear need, therefore, for identification and characterization of proteins which modulate chromatin structure, both normally and in disease states. In particular, there is a need to specifically, and in a controlled manner, manipulate chromatin structure and re-programming so as to effect expression of a gene or genes of interest in order to treat or prevent disease and/or to manipulate biological processes *in vivo* and *in vitro.*

[0019] **SUMMARY OF THE INVENTION**

[0020] The invention is directed, in one aspect, to a method of modulating chromatin structure, the method comprising altering expression of PARP-e.

[0021] The invention is also directed, in another aspect, to an isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of the nucleotide sequence of the DNA contained in Genbank Accession No. AF533701 or Genbank Accession No. AF533702. In another embodiment, the invention is directed to an isolated nucleic acid molecule comprising the nucleotide sequence of Genbank Accession No. AF533701 (SEQ ID NO: 1) or Genbank Accession No. AF533702 (SEQ ID NO: 10).

[0022] In another aspect, the invention is direct to an isolated PARP-e protein which comprises an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO: 2. In a different aspect, the invention is directed to an isolated protein comprising an amino acid

sequence at least 90% identical to amino acids 1 to 613 of SEQ ID NO. 2 wherein said protein has the activity of modulating chromatin structure.

[0023] In a yet a different aspect, the invention is directed to an isolated polynucleotide molecule selected from the group consisting of a) a polynucleotide molecule having at least 95% sequence identity to SEQ ID NO: 1; b) a polynucleotide molecule which is a fragment of a); and, c) a polynucleotide molecule which is the complementary nucleotide sequence of (a) or b).

[0024] In yet a different embodiment, the invention is directed to an isolated PARP-e protein having an amino acid sequence selected from the group consisting of: a) the amino acid sequence as set forth in SEQ ID NO. 2; and, b) the amino acid sequence encoded by a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO. 1; c) the amino acid sequence encoded by a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of the nucleotide sequence of the DNA contained in Genbank Accession No. AF533701 (SEQ ID NO: 1) or Genbank Accession No. AF533702 (SEQ ID NO: 10); and, d) the amino acid sequence encoded by an isolated nucleic acid molecule comprising the nucleotide sequence of Genbank Accession No. AF533701 (SEQ ID NO: 1) or Genbank Accession No. AF533702 (SEQ ID NO: 10).

[0025] The invention is also directed to a method of inhibiting the growth of an insect, comprising: a) creating an insertion mutation in the insect PARP-e gene of a first early insect embryo; b) culturing said first embryo to produce an insect of a first mutant strain; c) creating an insertion mutation in the insect PARP-e gene of a second early insect embryo; d) culturing said second embryo to produce an insect of a second mutant strain; e) mating an insect of said first mutant strain with an insect of said second mutant strain; wherein larvae that contain both said first and second mutations show inhibited growth as compared to an insect not comprising both said first and second mutations. In a different aspect, the invention is also directed to a method of inhibiting the growth of an insect, comprising: a) contacting embryonic insect cells with a composition comprising an effective amount of a dsRNA molecule specific for PARP-e, wherein contact with said dsRNA molecule inhibits growth of said insect.

[0026] **BRIEF DESCRIPTION OF THE FIGURES**

[0027] Figures 1 A-F. Structure and expression of the *Drosophila Parp* locus. (A) Deduced genomic structure of the 300 kb *Parp* region; open boxes are sequenced. The arrangement of the exons encoding Parp-I is shown above (Uchida *et al.* 1993; Hanai *et al.* 1998). Below, the position of three unlinked *Drosophila* genomic contigs (thin black lines: AE002935, AE002666

and AE002892) homologous to Parp-I exons are shown at right (Adams *et al.* 2000). Pm1 indicates the Parp-I promoter deduced from 5' cDNA sequences (Hanai *et al.* 1998). A single cDNA isolated from early ovarian stages, GM10715, comprises the 5' and 3' regions of the alternatively spliced Parp-e transcript. The 5'-most 273bp of GM10715 matches the genomic sequence flanking a P element insertion, *CH(3)1* (Zhang *et al.* 1994. *Proc. Nat. Acad. Sci. USA.* 91: 3539-3543). A map of this region (left portion of figure) was constructed by chromosome walking using a P1 genomic library (Kimmerly *et al.* 1996. *Genome Research. 6*: 414-430) (below, thick black lines). The two resulting scaffolds were sequenced and found to span four small pre-existing genomic sequence contigs (thin black lines) and to link to a fifth (AE003403). The color code indicates which portion of PARP is encoded: DNA binding (red), automodification (purple), catalytic (blue), non-coding (green and yellow). (B) Multiple *Parp* transcripts. A Northern blot of poly(A)-containing RNA from the indicated developmental stages reveals both a 3.2 kb RNA, the size predicted for Parp-I, and a 2.6 kb RNA, the approximate size expected for Parp-II and Parp-e. *Parp*-homologous RNAs are abundant in both ovaries and embryos, and are reduced but still detectable in second instar larvae and adults. (C) Whole mount *in situ* hybridization using a 1.4 kb cDNA probe from the DNA-binding domain common to all isoforms labels *Parp* RNA in nurse cells and in oocytes from stage 9-14 follicles. (D) RT-PCR using isoform-specific primers (see diagrams) that distinguish between Parp-I (or Parp-II) and Parp-e demonstrate that Parp-e is produced in ovaries and embryos, but not at detectable levels in 2nd instar larvae, or in adults outside the ovary. (E) Nuclei are shown from brains of third instar larvae expressing a Parp-I-DsRed fusion gene (see Examples). Protein is abundant in the chromocenter (C), the nucleolus (N) and at sites within euchromatin. (F) Third instar larval brain nuclei stained with anti-poly(ADP-ribose) antibody 10H show that protein-associated ADP-ribose is found in the same regions as PARP-DsRed.

[0028] Figures 2 A-B. DNA sequence of the heterochromatic region containing *Parp*. (A) A diagram summarizing the sequence organization of the region as determined from this study (see Examples) and from Adams *et al.* (2000) is shown. Genes defined by cDNAs sequenced as part of this study are shown in red (boxes are exons). The names of retrotransposons (black) and of transposons (blue) are given above the region of homology represented as an arrow (arrowhead - 3' end). Regions containing only small sequence blocks related to a particular transposon are indicated by parallel bars. The position of the *CH(3)1* insertion and the location of the putative *Parp* promoters Pm1 and Pm2 are indicated. Gaps in the sequence of known or estimated size are represented by hash marks. (B) An ideogram of chromosome 3 heterochromatin shows the cytological region of *CH(3)1* insertion (Zhang *et al.*1994). Below, a chromosome set from a

CH(3)1/ TM3 third instar larval neuroblast is shown that has been hybridized *in situ* with a *Parp* cDNA (green) and transposon-specific sequences (red). The partial overlap of the *Parp* and *CH(3)1* sequences indicates that *Parp* and *CH(3)1* are located near each other in 3R heterochromatin. (Note: the TOTO-3 used for this confocal micrograph does not reveal full morphological detail; but chromosomes were also scored using DAPI; *CH(3)1* was localized previously to h55 (Zhang *et al.* 1994)).

[0029] Figures 3 A-E. The *CH(3)1* complementation group disrupts *Parp* expression and activity. (A) Timelines of development of wild type (above) and *CH(3)1* homozygotes (below) are shown. The fraction of animals at each developmental stage are plotted as a function of time, revealing the strong developmental delay caused by *CH(3)1*. (B) Preparation of larval mouth hooks, which distinguish larval instars, are illustrated showing the characteristic appearance of the normal l2 mouthhooks (left) and of mouthhooks from *CH(3)1* mutants arrested at the onset of ecdysis 2 (right). (C) Northern blot of poly(A)-containing RNA from wild type larvae and four days old *CH(3)1* larvae showing reduced levels of *Parp* 3.2 kb mRNA. (D) Proteins labeled by ADP-ribosylation in wild type (wt) and *CH(3)1* mutant larvae. An autoradioagram of a gel of ^{32}P-labeled protein is shown (see Examples). The prominent band at 117 kd in the wild type has the expected molecular weight of PARP itself. Stained protein in a segment of the same gel is shown as a loading control. (E) RNAi treatment of embryos eliminates detectable *Parp* mRNA in 16hr embryos and larvae. An RT-PCR assay recognizing all forms of the *Parp* transcripts is shown; primers specific for the alcohol dehydrogenase gene (*Adh*) gene serve as a loading control.

[0030] Figures 4 A-C. *Parp* mutations or *Parp* (RNAi) elevate copia transcript levels. (A) A Northern blot of total RNA from 2nd instar larvae of the indicated genotypes was probed with copia sequences. The 5.5 kb copia transcript is overproduced up to 50-fold in *CH(3)1* or *CH(3)4* homozygotes, and in *CH(3)1/CH(3)4* trans-heterozygotes compared to wild type. An *rp49* probe was used as a loading control. (B) Quantitative RT-PCR shows that injection of *Parp*-specific RNAi, but not buffer, causes copia RNA to be overproduced. Primers specific to Adh served as a loading control. (C) copia RNA accumulation does not cause lethality. Injection of mutant *CH(3)1* embryos with RNAi specific to copia suppressed the accumulation of excess copia RNA and resulted in the elimination of all copia transcripts detectable by RT-PCR within 16 hours. Sequential dilutions of the RNAi gave a graded response. However, the treatment did not rescue larval lethality.

[0031] Figures 5A-D. *Parp* mutations alter nuclear morphology and chromatin accessibility to nuclease. (A) DAPI stained nuclei from 2nd instar larval salivary glands of wild type (upper) or

CH(3)1 mutants (lower). A single nucleus is presented at higher magnification in the insets. Nuclei in the mutant appear more diffuse, have a less distinct chromocenter and lack the region of low DNA density caused by the presence of a normal nucleolus. (B) Nuclei from *CH(3)1* mutant larvae were treated with increasing concentrations of micrococcal nuclease (triangles) prior to DNA extraction, digestion with PstI and analysis on Southern blots probed with a copia or GATE probe. Pst digestion produces no small internal fragment of copia or GATE resolvable within the molecular weight range of the gel. At all concentrations, retrotransposon specific sequences were far more sensitive to digestion in the mutant. (C) The same analysis as in (B) was carried out using nuclei at the indicated times after injection of *Parp*-specific RNAi. copia sequences from RNAi-injected animals become increasingly sensitive to micrococcal nuclease digestion at increasing time after RNAi injection, compared to buffer injected controls (C). (D) Micrococcal nuclease assays were carried out as in (B) and analyzed with a probe from the *Parp* gene region encoding exons 3, 4 and 5, and with probes specific for the single copy euchromatic genes *actin 5C* and *rp49*. *Parp* sequences are much more accessible to digestion in the mutant, including a band containing exon 3 and Pm1 (asterisk). To ensure that experiments with heterochromatic and single copy probes were comparable, the same blot was used for copia, GATE, actin 5C and rp49. The blot assayed with Parp in (D) was re-probed with copia as a control and showed the same differential digestion as in (B).

[0032] Figures 6A-D. Expression of Parp-I or Parp-e cDNA rescues defects in *CH(3)1* mutants. (A) Partial restoration of normal nuclear morphology by expression of Parp-I. Immunofluorescent detection of the nucleolar antigen AJ1 (red) and DNA (green) is shown in larval salivary glands of the indicated genotypes. AJ1 staining alone is shown on the right. In *CH(3)1* mutants (center), AJ1 is cytoplasmic rather than in nucleoli as in wild type (left). Expression of Parp-I cDNA (right) restores nucleoli and nuclear AJ1 staining in a mosaic manner; note cells at the top of the figure with normal localization, but cells near the bottom still show a mostly cytoplasmic distribution of AJ1 reactivity. (B) A Northern blot of RNA from larvae of the indicated genotypes shows that Parp-e cDNA expression greatly elevates the level of 2.6 kb Parp-e mRNA and also of the 3.2 kb Parp-I mRNA. Note that copia-specific RNA accumulation is greatly reduced in *CH(3)1* mutant larvae that express Parp-e cDNA. *rp49* hybridization serves as a loading control. (C) A Western blot of proteins isolated from larvae of the same genotypes as in (C), and probed with an antibody specific for poly(ADP-ribosyl) moieties. Expression of Parp-e cDNA in a *CH(3)1* homozygous background increases the amount of poly(ADP)-ribose-modified proteins to levels greater than in wild type. As in the

wild type, diverse protein areas are affected, the most prominent of which is the size of PARP-I itself (shown). An actin antibody is used as a loading control.

[0033] Figures 7 A-E. *Parp*^{CH1} and *Sir2*^{05327} have opposite dominant effects on the variegated expression of GAL4/UAS constructs. The variegated expression of an Arm-Gal4 driven UAS-Tim17B -DsRed construct (A-B) or a UAS-Sir2-DsRed construct (D-E) is modified by background genotype. In a *Parp*^{CH1} / + background (A and D), expression is strongly reduced compared to expression in a wild type background (B and E). Similar variegated expression of the same constructs driven by 69B-GAL4 is almost completely suppressed in a *Sir2*^{05327}/+ background (C and F). Green = DNA.

[0034] Figures 8A-J. PARP is distributed widely in chromatin while ADP-ribose-modified proteins are enriched in polytene chromosome puffs. (A) In these diploid l3 larval brain cells, PARP-DsRed (red) is abundant in nucleoli (arrow) located near the chromocenter (arrowhead) but is also found throughout the nucleus. (DNA = green). (B) Nucleoli in l3 larval gastric cells labeled with Fibrillarin (red). Compare normal structure (arrow) in wild type (B) with variegated presence of nucleoli (arrow) in Parp^{CH1} (C). (D) PARP-DsRed is abundant in nucleoli and is present a lower levels along the chromosomes of this l3 salivary gland cell. (E) The pattern of incorporated biotinylated-NAD 3 hours after injection of an l3 larvae shows heavy incorporation in the nucleolus (arrow), at certain euchromatic sites, and low labeling generally along the chromosomes (arrowhead). (F) Poly(ADP-ribosyl)ated proteins are enriched in the nucleolus (arrow) and at discrete sites within euchromatin (arrowhead) in this l3 salivary gland nucleus. (G) A nucleus similar to that shown in (F) was squashed, revealing abundant poly(ADP-ribosyl) modified proteins (yellow) within polytene chromosome puffs (arrow indicates the 2B puff; arrowhead indicates 74A, 75B puffs). (DNA = purple). (H,J) and I all show a short section of chromosome 3L containing the region of the major early Edison puffs 74A and 75B. Prior to the induction of puffing late in l3, the level of poly(ADP-ribosyl) modified proteins (red) are normal (H), but elevated levels are always observed after the puff has formed (I) (DNA = blue). The amount of PARP-EGFP (green) in this region is similar to that found all along the chromosome (J).(DNA= red).

[0035] Figures 9A-H. Parp is required for heat shock puffing and gene expression. The site of the major heat shock puffs containing hsp70 genes at 87A and 87C are shown from larvae that had not been heat shocked (A) and following 30 minutes at 37 °C (B). (C) Poly(ADP-ribosyl) modified proteins are present at general levels prior to heat treatment (0°), but increase greatly within ten minutes following 37 °C treatment of l3 larvae. Twenty minutes after the heat shock the amount of staining is decreasing (D). (E) When heat shocks were given 30 minutes after

injecting larvae with the PARP inhibitor 3-aminobenzamide, no increase in poly(ADP-ribosyl) modified proteins occurs. (F) 87A, 87C heat shock puff pairs of various sizes (3 are shown) can be visualized in salivary gland chromosomes from l2 larvae using antibody to RNA polymerase. (G) A histogram comparing the size of the 87A, 87C puff pairs in l2 larvae from wild type (black) or in $Parp^{CH1}$ (red). (H) A Western blot comparing the amount of heat shock-induced Hsp70 protein produced in wild type (wt) or in $Parp^{CH1}$ larvae. Hsp70 production was reduced 5-10 fold when the blot was normalized using Actin.

[0036] Figures 10A-D. PARP is required to express innate immunity genes. (A) Cells from $Parp^{CH1}$ animals frequently become infected with bacteria as revealed by DAPI staining. (B) Variegated PARP expression is shown by the presence or absence of AJ1-stainined nucleoli (red). (C) Infection with bacteria (arrow) is only found in cells that lack PARP activity as indicated by the absence of nucleoli. (D) A Western blot quantitating the response of wild type (wt) and in $Parp^{CH1}$ larvae to infection with injected *E. coli* bacteria. The levels of the NF-κB-dependent innate immunity genes Diptericine and Drosomycin were quantitated using the fusion genes and antibodies directed at the reported epitopes.

[0037] Figure 11. Model of PARP-mediated chromatin re-modeling. A model of the proposed role of PARP in 1) receiving a local signal activating the enzymatic activity; 2) modifying nearby chromosomal proteins so that they dissociate from the DNA; 3) following cleavage, the original proteins, some of which may have become newly modified and formed new complexes with novel proteins, reassemble to form a specifically modified chromatin state.

[0038] Figure 12. Nucleotide sequence of PARP-e cDNA (SEQ ID NO: 1).

[0039] Figure 13. Amino acid sequence of PARP-e protein (SEQ ID NO: 2). The amino acid sequence was derived from the cDNA sequence.

[0040] Figure 14. Nucleotide sequence of PARP DNA (SEQ ID NO: 10).

[0041] **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

[0042] Definitions

[0043] As is generally the case in biotechnology, the description of the present invention herein has required the use of a substantial number of terms of art. Although it is not practical to do so exhaustively, definitions for some of these terms are provided here for ease of reference. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Definitions for other terms also appear elsewhere herein. However, the definitions provided here and elsewhere herein should always be considered in determining the intended scope and meaning of the defined terms. Although any methods and materials similar or equivalent to

those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

[0044] Euchromatin. As used herein, "euchromatin" refers to transriptionally active genes and is loosely packed.

[0045] Heterochromatin. As used herein, "heterochromatin" is a form of condensed chromatin and is a complex of histones, DNA, and other proteins. Heterochromatin remains in a condensed form throughout the entire life cycle, and contains transcriptionally inactive DNA.

[0046] Isoform. As used herein, an "isoform" refers to a protein produced from a single gene by alternative mRNA splicing.

[0047] Positional effect variegation. As used herein, the term "positional effect variegation" refers to the effect obtained when genes that are transposed adjacent to heterochromatic regions undergo transcriptional silencing in only some cells in a population. The effect is inheritable in an epigenetic manner.

[0048] Stringent Hybridization Conditions. As used herein, the term "stringent hybridization conditions" means overnight incubation at 42° C. in a solution comprising: 50% formamide, 5 X SSC (1 X SSC =150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 X Denhardt's solution, 10% dextran sulfate, and 20 u/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1 X SSC at about 65° C., or equivalent conditions. Equivalent conditions are easily determined by one of ordinary skill in the art using methods and materials publicly known and available.

[0049] Variegation. As used herein, the term "variegation" refers to a change in phenotype due to mutation during somatic development.

[0050] Wild-type enzyme. As used herein, the term "wild-type enzyme" refers to an enzyme that will be active at a level of activity found in nature and typically comprises an amino acid sequence found in nature.

[0051] The invention is directed, in one aspect, to a method of modulating chromatin structure, the method comprising altering expression of PARP-e. In one embodiment of the method, when expression of PARP-e is increased, the expression of PARP-1 is increased. In another embodiment of the method, when expression of PARP-e is decreased, the expression of PARP-1 is decreased. In a different embodiment, the chromatin is present in a eukaryotic cell. In another embodiment, the chromatin is present in a plant cell. In a preferred embodiment, the chromatin is present in an animal cell. In a highly preferred embodiment the cell is an embryonic cell. In another highly preferred embodiment, the cell is a stem cell.

[0052] In another embodiment of the method, the chromatin structure is selected from the group consisting of heterochromatin and repetitive sequences. In a preferred embodiment, modulation of chromatin structure results in gene activation. In a different preferred embodiment, modulation of chromatin structure results in gene repression. In a highly preferred embodiment, the increased PARP-e expression effects chromatin decondensation. In another highly preferred embodiment, the decreased PARP-e expression effects chromatin condensation.

[0053] The invention is also directed, in another aspect, to an isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of the nucleotide sequence of the DNA contained in Genbank Accession No. AF533701 (SEQ ID NO: 1) or Genbank Accession No. AF533702 (SEQ ID NO: 10). In another embodiment, the invention is directed to an isolated nucleic acid molecule comprising the nucleotide sequence of Genbank Accession No. AF533701 (SEQ ID NO: 1)or Genbank Accession No. AF533702 (SEQ ID NO: 10).

[0054] In another aspect, the invention is direct to an isolated PARP-e protein which comprises an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO: 2. In a different aspect, the invention is directed to an isolated protein comprising an amino acid sequence at least 90% identical to amino acids 1 to 613 of SEQ ID NO. 2 wherein the protein has the activity of modulating chromatin structure.

[0055] In a preferred embodiment, the invention is directed to an isolated polynucleotide molecule selected from the group consisting of a) a polynucleotide molecule having at least 95% sequence identity to SEQ ID NO: 1; b) a polynucleotide molecule which is a fragment of a); and, c) a polynucleotide molecule which is the complementary nucleotide sequence of (a) or b). In one embodiment, the isolated polynucleotide molecule has SEQ ID NO: 1. In another embodiment, the invention is directed to an isolated polynucleotide molecule comprising the polynucleotide having SEQ ID NO: 1.

[0056] In yet a different embodiment, the invention is directed to an isolated PARP-e protein having an amino acid sequence selected from the group consisting of: a) the amino acid sequence as set forth in SEQ ID NO. 2; and, b) the amino acid sequence encoded by a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO. 1; c) the amino acid sequence encoded by a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of the nucleotide sequence of the DNA contained in Genbank Accession No. AF533701 (SEQ ID NO: 1)or Genbank Accession No. AF533702 (SEQ ID NO: 10); and, d) the amino acid sequence encoded by an isolated nucleic acid molecule comprising the nucleotide

sequence of Genbank Accession No. AF533701 (SEQ ID NO: 1) or Genbank Accession No. AF533702 (SEQ ID NO: 10).

[0057] The invention is also directed to a method of inhibiting the growth of an insect, comprising: a) creating an insertion mutation in the insect PARP-e gene of a first early insect embryo; b) culturing said first embryo to produce an insect of a first mutant strain; c) creating an insertion mutation in the insect PARP-e gene of a second early insect embryo; d) culturing said second embryo to produce an insect of a second mutant strain; e) mating an insect of said first mutant strain with an insect of said second mutant strain; wherein larvae that contain both said first and second mutations show inhibited growth as compared to an insect not comprising both said first and second mutations. In a different aspect, the invention is also directed to a method of inhibiting the growth of an insect, comprising: a) contacting embryonic insect cells with a composition comprising an effective amount of a dsRNA molecule specific for PARP-e, wherein contact with said dsRNA molecule inhibits growth of said insect. In a preferred embodiment, the insect is a Drosophila fly.

[0058] The role of PARP on gene expression during the life cycle is studied herein. PARP protein is found throughout chromosomes, but poly(ADP-ribose)-modified proteins are enriched in polytene chromsome puffs, suggesting that PARP is differentially active in these regions. ADP-ribosylated proteins accumulate immediately following heat shock at the 87A and 87C puffs. In *Parp* mutant larvae, heat shock-induced puffing at these sites and Hsp70 production was strongly reduced. Bacterial infection induces elevated levels of ADP-ribosylation at certain chromosome sites. *Parp* mutants are abnormally susceptible to bacterial infection, and fail to normally activate Drosopterin and Diptericin, two NF-κB-dependent innate immune response genes. These observations support the idea that PARP plays a critical part in remodeling chromatin at a wide variety of times during Drosophila development as well as in response to environmental stresses including DNA damage. Puffs may be a physical manifestation of this type of chromatin-based transcriptional activation.

[0059] Mutations in the heterochromatic *Parp* gene have been characterized. Rather than simply functioning as a repair enzyme, *Parp* is necessary for viability and to organize the chromatin structure of nucleoli, heterochromatin and other sequences during development. Reduction of *Parp* function causes hyperexpression of the copia retrotransposon and enhances the variegation of GAL4 transgenes. Studies herein show that *Parp* plays a fundamental role in organizing chromatin structure during *Drosophila* development, and suggest that ADP-ribosylation of chromosomal proteins plays an important role in chromatin remodeling.

[0060] **Proteins and Polypeptides**

[0061] The present invention relates to a PARP-e protein which has the deduced amino acid sequence of SEQ ID NO:2 as well as fragments, analogs and derivatives of such polypeptide. The terms "fragment, " "derivative" and "analog" when referring to the polypeptide of SEQ ID NO:2 means a polypeptide which retains essentially the same biological function or activity as the polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide. The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

[0062] The fragment, derivative or analog of the polypeptide of SEQ ID NO:2 may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and the substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide, or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art using the teachings herein.

[0063] The polypeptides of the present invention include the polypeptide of SEQ ID NO:2 as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO:2 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the polypeptide of SEQ ID NO:2 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

[0064] As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide. Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides.

[0065] The invention further provides an isolated Parp-e peptide having the amino acid sequence encoded by the cDNA (SEQ ID NO: 1), or the amino acid sequence in SEQ ID NO:2, or a peptide or polypeptide comprising a portion of the above polypeptides. It will be

recognized in the art that some amino acid sequences of the peptide can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity.

[0066] Thus, the invention further includes variations of the PARP-e protein which show substantial activity or which include regions of partial peptide activity such as the protein portions discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions. As indicated above, guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie *et al., Science* 247:1306 (1990).

[0067] As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein. Amino acids in the PARP-e protein of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham *et al.* 1989. *Science* 244:1081-1085). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as receptor binding or *in vitro*, or *in vivo* proliferative activity. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith *et al.* 1992. *J. Mol. Biol.* 224:899-904 and de Vos *et al.,* 1992. *Science* 255:306-312).

[0068] The polypeptides of the present invention are preferably provided in an isolated form. By "isolated polypeptide" is intended a polypeptide removed from its native environment. Thus, a polypeptide produced and/or contained within a recombinant host cell is considered isolated for purposes of the present invention. Also intended as an "isolated polypeptide" are polypeptides that have been purified, partially or substantially, from a recombinant host cell.

[0069] By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a peptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the peptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those

terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

[0070] As a practical matter, whether any particular polypeptide is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in SEQ ID NO:2 can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

[0071] The accession numbers, and the nucleotide sequences which they designate, are available through publicly accessible genomic data bases such as GenBank, and the Berkeley Drosophila Genome Project. The sequence of the polynucleotides contained in the accession numbers, as well as the amino acid sequence of the polypeptides encoded therefrom, are incorporated herein by reference.

[0072] **Polynucleotides and Nucleic Acid Molecules**

[0073] Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the Model 373 from Applied Biosystems, Inc.), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

[0074] Isolated nucleic acid molecules of the present invention include, for example, the DNA molecule shown in SEQ ID NO:1; DNA molecules comprising the coding sequence for the Parp-e protein; and DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode the Parp-e protein. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate such degenerate variants.

[0075] The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By a fragment of an isolated nucleic acid molecule having the nucleotide sequence of a DNA encoding PARP-e or the nucleotide sequence shown in SEQ ID NO:1 is intended fragments at least about 15 nt, and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably, at least about 40 nt in length which are useful as diagnostic probes and primers as discussed herein. Of course, larger fragments 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, or 323 nt in length are also useful according to the present invention as are fragments corresponding to most, if not all, of the nucleotide sequence of a DNA encoding the PARP-e protein or as shown in SEQ ID NO:1. By a fragment at least 20 nt in length, for example, is intended fragments which include 20 or more contiguous bases from a nucleotide sequence of a DNA encoding PARP-e or the nucleotide sequence as shown in SEQ ID NO:1.

[0076] In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule described above, for instance, SEQ ID NO: 1 or SEQ ID NO: 10.

[0077] **Hybridization**

[0078] A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (see Sambrook *et al.*, supra). The conditions of temperature and ionic strength determine the "stringency" of the hybridization.

[0079] For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m of 55° can be used, e.g., 5 X SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5 X SSC, 0.5% SDS. Moderate stringency hybridization conditions correspond to a higher T_m, e.g., 40% formamide, with 5 X or 6 X SCC. High stringency hybridization conditions correspond to the highest T_m, e.g., 50% formamide, 5 X or 6 X SCC. By "stringent hybridization conditions" is intended overnight incubation at 42 °C in a

solution comprising: 50% formamide, 5 X SSC (750 mM NaCl, 75 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 X Denhardt's solution, 10% dextran sulfate, and 20 ug/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1 X.SSC at about 65° C.

[0080] Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA.

[0081] For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (see Sambrook *et al.*, supra, 9.50-9.51). For hybridization with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook *et al.*, supra, 11.7-11.8). Preferably a minimum length for a hybridizable nucleic acid is at least about 12 nucleotides; preferably at least about 16 nucleotides; and more preferably the length is at least about 24 nucleotides; and most preferably 36 nucleotides.

[0082] Fragments of the PARP-e gene (SEQ ID NO: 1) may be used as a hybridization probe for a cDNA library to isolate the full length cDNA and to isolate other cDNAs which have a high sequence similarity to the PARP-e gene or encode a protein having similar biological activity. Probes of this type preferably have at least 30 bases and may contain, for example, 50 or more bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete PARP-e gene including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to the PARP-e gene are used to screen a library of cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to. The screen is not limited to use in any particular type of organism, all that is required is that the organism have nucleic acids.

[0083] The present invention further relates to polynucleotides which hybridize to the above described sequences if there is at least 70%, preferably at least 90%, and more preferably at least

95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the polynucleotides described herein. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the polynucleotides described herein in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the polypeptide encoded by the cDNA of SEQ ID NO:1 or the polypeptide having SEQ ID NO: 2.

[0084] By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed herein.

[0085] By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., the nucleotide sequence as shown in SEQ ID NO:1).

[0086] The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the PARP-e protein. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

[0087] Such variants include those produced by nucleotide substitutions, deletions or additions, which may involve one or more nucleotides. The variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the Parp-e protein or portions thereof. Also especially preferred in this regard are conservative substitutions.

[0088] By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding a PARP-e protein is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the protein. In other words, to obtain a polynucleotide

having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

[0089] As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in SEQ ID NO:1 or to the nucleotide sequences of GenBank accession nos. AF533701 and AF533702, can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2: 482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

[0090] The present application is directed to nucleic acid molecules at least 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in SEQ ID NO:1 or to the nucleotide sequences of GenBank accession nos. AF533701 and AF533702, irrespective of whether they encode a polypeptide having Parp-e activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having Parp-e activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having Parp-e activity include, for example, (1) isolating the Parp-e gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization to metaphase chromosomal spreads to provide precise chromosomal location of the Parp-e protein gene (Verma *et al.*, Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York (1988)); and Northern Blot analysis for detecting Parp-e mRNA expression in specific tissues. Preferred, however, are nucleic acid molecules having sequences at least 95%, 96%, 97%, 98% or 99% identical to a nucleic acid sequence shown in SEQ ID NO:1 or to the

nucleic acid sequence of GenBank accession nos. AF533701 (SEQ ID NO: 1) and AF533702 (SEQ ID NO: 10), which do, in fact, encode a protein having Parp-e protein activity

[0091] Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 95%, 96%, 97%, 98%, or 99% identical to a nucleic acid sequence of GenBank accession nos. AF533701 (SEQ ID NO: 1) or AF533702 (SEQ ID NO: 10), or to a nucleic acid sequence shown in SEQ ID NO:1 will encode a polypeptide having Parp-e protein activity. Since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having Parp-e protein activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

[0092] Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a Parp-e gene may be used in the practice of the present invention including those comprising conservative substitutions thereof. These include but are not limited to modified allelic genes, modified homologous genes from other species, and nucleotide sequences comprising all or portions of Parp-e genes which are altered by the substitution of different codons that encode the protein.

[0093] **Standard techniques**

[0094] Standard techniques for cloning, DNA isolation, amplification and purification, for enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like, and various separation techniques are those known and commonly employed by those skilled in the art. A number of standard techniques are described in Sambrook *et al.* (1989) Molecular Cloning, Second Edition, Cold Spring Harbor Laboratory Press, Plainview, New York; Maniatis *et al.* (1982) Molecular Cloning, Cold Spring Harbor Laboratory Press, Plainview, New York; Wu (ed.) (1993) Meth. Enzymol. 218, Part I; Wu (ed.) (1979) Meth Enzymol. 68; Wu *et al.* (eds.) (1983) Meth. Enzymol. 100 and 101; Grossman and Moldave (eds.) Meth. Enzymol. 65; Miller (ed.) (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Old and Primrose (1981) Principles of Gene Manipulation, University of California Press, Berkeley; Schleif and Wensink (1982) Practical Methods in Molecular Biology; Glover (ed.) (1985) DNA Cloning Vol. I and II, IRL Press, Oxford, UK; Hames and Higgins (eds.) (1985) Nucleic Acid Hybridization, IRL Press,

Oxford, UK; and Setlow and Hollaender (1979) Genetic Engineering: Principles and Methods, Vols. 1-4, Plenum Press, New York. Abbreviations and nomenclature, where employed, are deemed standard in the field and commonly used in professional journals such as those cited herein.

[0095] EXAMPLES

[0096] Example 1. *Drosophila* strains and genetics

Genetic markers are described in Flybase (1999) (FlyBase 1999. *Nucleic Acids Res.* 27: 85-88) and stocks were obtained from the Bloomington Stock Center except as indicated. The *CH(3)1* and *CH(3)4* strains were generated in a single P-element mutagenesis screen (Zhang et al. 1994). *CH(3)1* was found to be viable in combination with Df(3R)10-65; Southern blotting indicated that this deletion does not remove PARP coding sequences (data not shown). *y* $w^{67c23(2)}$ was used as the host for transformation. The driver strain for the rescue experiments was P{GAL4-arm.S) (armGAL4)} (Sanson *et al.* 1996. *Nature.* **383:** 627-630). The 69B GAL4 driver is described by Brand (Brand *et al.* 1993. *Development.* **118:** 401-415). $Sir2^{05237}$ was constructed by Karpen *et al.* (Karpen *et al.* 1992. *Genetics.* **132:** 737-753) and is described by Rosenberg and Parkhurst (2002). Balancer chromosomes carrying Kr-GFP were used to differentiate homozygous mutant embryos and larvae:TM3, Sb, P{w^+, Kr-GFP} and CyO, P{w^+, Kr-GFP} (Casso *et al.* 2000. *Mech. Dev.* **91:** 451-454). Imprecise excision of *CH(3)1* was carried out as described previously (Zhang *et al.* 1994). Transformation experiments were carried out as described in Tulin *et al.* (2002) using the y $w^{67c23(2)}$ strain as host.

[0097] Example 2. Developmental Timing Measurements

[0098] Embryos were collected on grape juice/agar plates for 2 hours at 25°C, aged 10-12 hours, and subsets were subsequently analyzed each 1-2 hours during daytime for 4-8 days. Larval stages were identified by mouth hook and/or posterior appendage morphology.

[0099] Example 3. Construction of transgenic *Drosophila*

[00100] For the rescue experiments, pP{w+, UAST-PARP-I} was constructed by cloning the NotI/KpnI fragment encoding PARP-I from cDNA LD02455 into the pUAST vector. pP{w+, UAST-PARP-e} was constructed by fusing a NotI/KpnI fragment encoding PARP-e from cDNA GM10715 into pUAST. To detect protein localization *in vivo*, PARP-I cDNA was fused to DsRed (Clontech Laboratories) in pP{w+, UAST-PARP-I-DsRed} and PARP-e was fused to EGFP (Clontech Laboratories) in pP{w+, UAST-PARP-e-EGFP}. To study the variegation of UAS constructs we built pP{w+, UAST-Tim17b-DsRed}, which contains a Tim17b cDNA fused in frame to DsRed (Clontech Laboratories) in pUAST. Transformation was as described

(Spradling *et al.* 1982. *Science.* 218: 341-347), with modifications (Prokhorova *et al.* 1994. *Genetika (Moscow).* 30: 874 - 878).

[00101] Example 4. Genomic mapping and sequencing

[00102] A physical map of the *Parp* region was constructed using cDNA libraries (Rubin *et al.* 2000. *Science* **287:** 2222-2224) and a P1 library (Kimmerly *et al.*1996) from the Berkeley *Drosophila* Genome Project (BDGP). Clone DS09016 was subcloned into pTZ19R using XbaI or EcoRI digestion and sequenced. The following cDNAs were sequenced and used to express PARP isoforms: LD02455 (Parp-I) and GM10715 (Parp-e, SEQ ID NO: 1). In addition, 13 other *Parp* cDNAs were fully sequenced. To determine the location of transcribed exons in the *Parp* region (Fig. 2) we also fully sequenced the following cDNAs: SD15682 (TK), RE01394 (Tim23), CK01513 (Tim17b) and LP01513 (no ORF).

[00103] Example 5. Fluorescent *in situ* hybridization (FISH)

[00104] Mitotic chromosomes were prepared as described by Laverty (web site: fruitfly.org/methods/cytogenetics). Probe preparation by nick translation, pretreatment, hybridization and signal detection were performed as previously described (Dej *et al.* 1999. *Development* **126:** 293-303). cDNA LD02455 was used to detect Parp coding sequences, and the PZ element without rosy gene sequences (Karpen and Spradling, 1992) was used to detect the PZ insertion in *CH(3)1*.

[00105] Example 6. Double stranded RNA interference (dsRNAi)

[00106] RNAi was prepared as described by Kennerdell (Kennerdell *et al.* 1998. *Cell* **95:** 1017-1026). The following regions were targeted: 269-864 for GM10715, 1-604 for LD02455 and 485-891 for copia. DsRNAi was injected into the posterior region of precellular blastoderm embryos at a concentration 0.5 ug/ul and the embryos were allowed to develop for an appropriate period under oil in a humid chamber prior to analysis.

[00107] Example 7. RT-PCR and Northern blot

[00108] Total RNA was isolated using Trizol reagent (Gibco BRL), precipitated twice with 3M LiCl, treated with Amplification Grade Dnase I (Gibco BRL) and poly(A)-containing RNA purified using a MessageMarker kit (Gibco BRL). The SuperScript Preamplification System (Gibco BRL) was then used to synthesize cDNA and for RT-PCR. The following primers were used to distinguish PARP-I and PARP-e transcripts: PI (5'-aaataataaatgtcttgaaattg-3') (SEQ ID NO: 3) for PARP-I, PIII (5'-gtcttgattttgtgtataccg-3') (SEQ ID NO: 4) for PARP-e and R4 (5'-ttttatgaaaccaattcg-3')(SEQ ID NO: 5) for both. Total Parp transcripts were detected using: D1 (5'-gtgtcgtggatgtgaac-3') (SEQ ID NO: 6) and R2 (5'-ttggaattctggattttg-3')(SEQ ID NO: 7) which target a common coding region within the DNA binding domain. Copia-specific

transcripts were detected using: 5'-copia (5'-ccgtttgatggcgagaagtacgcgatttgg-3)' (SEQ ID NO: 8) and 3'-copia (5'-ccatcgtaacacgaaggcaatgtgatc-3') (SEQ ID NO: 9) which target part of ORF1. For Northern blot analysis, at least of 2.5 ug of poly(A) RNA from second instar larvae was used per each lane. The PARP probe was from the DNA binding domain, while an *rp49* probe was used as a control.

[00109] Example 8. Nuclease sensitivity assays

[00110] Embryos were collected on grape juice/agar plates for 2 hours at 25^{0}C, aged for 12 hours or an appropriate period. The micrococcal nuclease sensitivity of purified nuclei was determined as described by Wu (Wu.1989. *Methods Enzymol.* 170: 269-289) and Quivy (Quivy *et al.* 1997. *Methods.* 11: 171-179) with minor modifications. Controls showing the absence of endogenous nuclease activity were carried out, and the levels of micrococcal nuclease used were calibrated for each stock.

[00111] Example 9. Immunohistochemistry and fluorescence microscopy

[00112] Tissues were fixed and stained with primary and secondary antibodies as described previously (Grieder *et al. Development.* 127: 4253-4264) and examined by confocal microscopy using a Leica TCS-NT microscope. Primary antibodies were: mouse monoclonal (mAb) Aj1 (1:100) and anti-fibrillarin (1:200) (from J. Gall); and mouse mAb 10H (1:20-50) from Dr. Manfred Frey (Steinbeis-Transferzentrum fur Angewandte Biologische Chemie). 10H specifically recognizes ADP-ribose polymers (Kawamitsu et al. 1984. *Biochemistry.* 23: 3771-3777). Nuclear staining by 10H such as that shown in Fig.8F was abolished in *Parp* mutant larvae, further confirming the specificity of this reagent (data not shown). Mouse Alexa-568 (Molecular Probes) (1:400) was used as a secondary antibody.

[00113] Example 10. pADPr assay

[00114] Embryos were collected and nuclei purified as for the nuclease sensitivity assay. Nuclei were incubated in nuclear buffer (Quivy 1997) containing 0.1mCi/ml of [^{32}P]-NAD (Amersham) for 15 minutes at room temperature. Then nuclei were washed twice in nuclear buffer, collected by centrifugation, preheated for 3 minutes. The protein gel was processed, dried and subjected to autoradiography.

[00115] Example 11. Antibodies and microscopy

[00116] Primary antibodies were: mouse monoclonal (mAb) Aj1 (1:100) and anti-fibrillarin (1:200) (from J. Gall); and mouse mAb 10H (1:20-50) from Dr. Manfred Frey (Steinbeis-Transferzentrum fur Angewandte Biologische Chemie). Anti-poly(ADP-ribose) antibody was obtained from Mouse Alexa-568 (Molecular Probes) (1:400). Rabbit polyclonal anti-fibrillarin antibody labels nucleoli; mouse mAb h10 (Steinbeis-Transferzentrum fur Angewandte

Biologische Chemie) recognizes the branch sites of (ADP-ribose) polymers; anti-actin (Sigma) recognizes Drosophila actin; rabbit polyclonal anti-GFP (Promega) recognizes PARP-EGFP in fixed tissue, and Drosomycin-GFP on Western Blots (Jung et al. 2000. Biotechniques 30: 594), mouse monoclonal anti-lacZ (Promega) recognizes Diptericine-lacZ; anti-Hsp70 recognizes Hsp70 on Western blots; Biotinylated NAD (Trevigen) was detected with avidin-rhodamine (Roche). 3-AB (Sigma) was used at a concentration of 2.5 mM.

[00117] Tissues were fixed and stained with fluoresceinated antibodies as described previously (Grieder *et al.* 2000) and examined by confocal microscopy using a Leica TCS-NT microscope.

[00118] **Example 12. Construction of PARP-I-dsRed andConstruction of PARP-e-EGFP**

[00119] The construction of flies expressing PARP-EGFP or PARP-DsRed was described previously and these lines gave identical pattersn of fluorescence (Tulin *et al.* 2002). Drosomycin -GFP and Diptericine-lacZ reporter genes are described (Jung *et al.* 2000). Oligreen (Molecular Probes) and propidium iodide (Sigma) were used to stain DNA. Transformation of Drosophila embryos was as described (Spradling *et al.* 1982), with modifications (Prokhorova *et al.* 1994).

[00120] **Example 13. Infection assay**

[00121] Embryos were challenged by injecting a sublethal dose of approximately 2 x 10^4 *E. coli* bacteria or a similar volume of sterile buffer. Survival of the injected animals was followed through adulthood and a fraction of the animals were removed and the presence of bacteria in the haemolymph and in tissues was studied by DAPI staining and microscopic examination.

[00122] **Example 14. Polytene chromosome analysis**

[00123] The protocol of Lavrov *et al.* was used to prepare polytene chromosomes for antibody staining, except that fixative included 10% trichloroacetic acid to block PARP glycohydrolase activity when the h10 antibody was to be used. Polytene chromosomes from second instar larvae were prepared as described by Paro *et al.* to preserve chromosomal proteins prior to antibody binding. Following binding of primary Ab, they were washed and secondary Ab was added. Finally, the chromosomal DNA was labeled with TODO3 and the preparations were examined using a Leica NTS.

[00124] **Example 16. Heat shock protocol for puff experiments**

[00125] PARP inhibitors: GPI 6150, 3-aminobenzamide, DPQ; PJ34 = N-(6-oxo-5,6-dihydro-phenanthridin-2-yl) -N,N-dimethylacetamide (Inotek Corp)., TZM, NU1025.

[00126] **RESULTS**

[00127] **Sequences encoding a novel PARP isoform are expressed in embryos**

[00128] The previously determined structure of the genomic region encoding Parp-I is shown on the right in Fig. 1A (Hanai *et al.* 1998; Adams *et al.* 2000). To search for additional *Parp* transcripts, clones corresponding to 14 *Parp*-related EST sequences (Rubin *et al.* 2000) were analyzed, and GM10715, derived from an early ovarian RNA library, was found to differ from Parp-I. The complete sequence of GM10715 (SEQ ID NO: 1) was determined, revealing an additional 920 bp intron within exon 8 encoding the PARP catalytic domain as well as 287 bp of novel 5' sequence that splice into the first Parp-I exon (exon 3) about 40 bp downstream from its 5'-end but 6 bp before the AUG codon (Fig 1A). The PARP isoform predicted by GM10715, which we name PARP-e ("embryonic") (SEQ ID NO: 2), should lack enzymatic function since the new intron removes conserved amino acids essential for catalytic activity including the NAD binding site.

[00129] Previous studies of Parp-I production showed that transcripts are abundant in late-stage ovarian follicles and embryos, but did not distinguish between transcripts encoding different isoforms (Hanai *et al.* 1998). We analyzed *Parp* expression throughout the *Drosophila* lifecycle using Northern blots (Fig. 1B), whole mount *in situ* hybridization (Fig. 1C) and RT-PCR with specific primers to distinguish Parp-e from Parp-I and II (Fig. 1D). The 3.2 kb Parp-I RNA and 2.6 kb Parp-II or Parp-e RNAs are expressed in ovaries, embryos and adults. In contrast to previous results, low levels of the 3.2 kb Parp-I mRNA remain in 2nd instar larvae (Fig. 1B). In the ovary, nurse cells express *Parp* RNA beginning as early as stage 4, while male germ cells strongly express *Parp* until the spermatid stage (not shown). Parp-e expression is detected only in adult ovaries and embryos (Fig. 1D).

[00130] To further analyze *Parp* expression, we constructed and expressed epitope-tagged versions of the two major PARP isoforms in flies. When expressed using this UAS/GAL4 based system, PARP-I (Fig. 1E) and PARP-e (not shown) are both highly enriched in nucleoli, heterochromatic chromosomal regions and diverse euchromatic sites in the cells of most embryonic and adult tissues. The distribution of ADP(ribosyl)-modified proteins, as revealed by immunostaining with an antibody specific for ADP-ribose polymers (Kawamitsu *et al.* 1984), was very similar, strongly labeling these same regions within nuclei (Fig. 1F). These experiments provide a clearer picture of developmentally regulated *Parp* expression and show a correlation between PARP protein and protein ADP(ribosyl) moieties.

[00131] ***Parp* spans a large region of 3R heterochromatin**

[00132] The structure of GM10715 implies that some *Parp* transcripts originate from a novel promoter(s), which we denote Pm2, located at or upstream from the GM10715 5' end. Using the isoform-specific 5'portion of GM10715 as a probe, six overlapping clones spanning

approximately 100 kb of genomic DNA flanking the 5' region of Parp-e were isolated from a *Drosophila* P1 genomic libraryand used to map and sequence this region (Fig 1A, Fig. 2). P1 clones were also recovered from the genomic region encoding Parp-I. At least 55 kb separates the upstream Parp-e sequence contig defined by DS09016 from the non-overlapping Parp-I 5' sequence contig AE002935. Sequence identity was also observed between the upstream region and the DNA flanking a previously described heterochromatic P element insertion, *CH(3)1* (Zhang *et al.* 1994). To confirm that the upstream region defined by GM10715 and *CH(3)1* really lies adjacent to PARP coding sequences, we showed that probes specific for PARP coding sequences and for the *CH(3)1* insertion generated overlapping in situ hybridization signals on metaphase chromosomes (Fig. 2B). The *CH(3)1* P element insertion was mapped previously to region h55-h56 of 3R heterochromatin (Zhang et al. 1994). Taken together, these studies define the structure of the *Parp* locus and confirm its location in the heterochromatin of chromosome 3R.

[00133] Analyzing the genomic DNA sequence surrounding the *Parp* transcription unit revealed several striking features of this 300 kb heterochromatic chromosome region (Fig 2A). About 210 kb (70%) lies within *Parp* and 4 other genes, including a thiamine kinase ortholog (*TK*) and two apparent mitochrondrial translocase subunits (*Tim17b* and *Tim23*). Most of the DNA within and surrounding the genes consists of transposons (blue) and retrotransposons (black) that are strikingly organized over the entire region studied. Nearly all are oriented in the same direction on the chromosome and opposite to the genes. These transposons have lost LTR homologies, and the *gypsy* elements lack insulator sequences that can disrupt enhancer-promoter interactions. Because unselected genomic sequences diverge rapidly during *Drosophila* evolution, our observations suggest that in recent evolutionary time the *Parp* region underwent extensive transposon invasion subject to some large-scale selective or mechanistic constraint on insertional orientation.

[00134] ***CH(3)1* defines a complementation group that disrupts *Parp* expression**

[00135] We characterized the *CH(3)1* strain to learn whether its recessive lethality (Zhang *et al.* 1994) is caused by disrupting *Parp* gene function. A second allele of the *CH(3)1* locus was found within another P element insertion strain, *CH(3)4,* but the *CH(3)4* P element cannot be responsible for the allelism as it is located on the opposite chromosome arm (3L) and mutates a different gene. However, both *CH(3)1* and *CH(3)1/CH(3)4* animals display a similar phenotype. Mutant *CH(3)1* homozygotes develop slowly and usually die during the second larval instar after 6-9 days (Fig 3A). Evidence of mitotic cell cycle defects was not seen; predominantly diploid larval tissues such as the brain are of normal size. However, examination of larval

mouth hooks shows that up to 50% of the mutant larvae are arrested at the onset of ecdysis II (Fig. 3B). When the *CH(3)1* element was imprecisely excised, about 7% of the derived chromosomes were homozygous viable and complemented *CH(3)4*, arguing strongly that the *CH(3)1* P element was responsible for the original lethality.

[00136] If *CH(3)1* alleles mutate *Parp*, then its gene transcripts should be reduced in the affected larvae. As predicted, we found that *Parp* expression is severely affected in both *CH(3)1* and *CH(3)1/CH(3)4* animals. 3.2 kb Parp-I mRNA levels are strongly reduced on Northern blots of RNA from mutant larvae (Fig. 3C) and using quantitative RT-PCR (not shown). ADP-ribosylation of proteins is also dramatically decreased in mutants (Fig. 3D). However, the effects observed on all forms of Parp were surprising since mutation of Pm2-initiated transcripts by the *CH(3)1* insertion might have been expected to only disrupt production of the 2.6 kb *Parp-e* mRNA and the enzymatically inactive PARP-e protein isoform. The small amount of *Parp* mRNA and enzymatic activity that does remain in the mutant larvae might come from transcripts initiated at Pm1 or from remaining maternal stores of *Parp* transcripts. To try and remove all *Parp* mRNA, we injected a 587 bp dsRNA specific for *Parp* into early embryos and observed that all traces of *Parp* mRNA detectable by RT-PCR were lost after 16 hours of embryonic development (Fig. 3E). More than 70% of the animals receiving *Parp* RNAi injections, unlike buffer-injected controls, arrested after hatching into first instar larvae, i.e., at an earlier point than in the mutants that retain low levels of residual *Parp* RNA. These observations further strengthen the connection between the *CH(3)1* locus and *Parp*.

[00137] **Loss of PARP derepresses the copia retrotransposon**

[00138] Because *Parp* is located in transposon-rich heterochromatin, we looked for effects of the mutation on transposon activity. We found that *CH(3)1, CH(3)4* and *CH(3)1/CH(3)4* animals dramatically overproduce the 5.5 kb transcript of the copia retrotransposon (Fig 4A). A similar large accumulation of copia-specific RNA was observed in embryos and larvae following injection of *Parp*-specific RNAi (Fig 4B), providing further support that the *CH(3)1* mutation acts directly on *Parp*. Tests using several other retrotransposable elements showed no increase in transcripts in *CH(3)1* mutant animals, so the increased expression appeared to be specific for copia. Copia hyper-expression in *CH(3)1* mutants and normal copia expression in their wild type sibs could be abolished by injecting copia-specific dsRNA into preblastoderm embryos (Fig. 4C) but *CH(3)1* lethality was not rescued. Thus, disrupting *Parp* expression causes copia hyper-expression, but this effect is not responsible for the lethal effects of *CH(3)1*.

[00139] **Disrupting *Parp* expression alters heterochromatin structure**

[00140] The *CH(3)1* mutation might affect a transcription factor that negatively regulates copia transcription or it might disrupt a protein that acts at the level of chromatin. We looked for global effects on chromatin by examining DAPI-stained nuclei from various tissues of *CH(3)1* homozygotes, and by carrying out nuclease sensitivity experiments. *CH(3)1* alleles dramatically alter nuclear morphology (Fig. 5A). DAPI-stained DNA from all mutant tissues examined appears more uniform than wild type, shows a less distinct chromocenter and lacks a nucleolar region of low DNA density. Copia chromatin is specifically affected, because copia-homologous sequences are much more sensitive to micrococcal nuclease digestion within *CH(3)1* mutant compared to wild type nuclei (Fig.5B). Even the lowest levels of nuclease, which digested very little copia-specific DNA in wild type, cleaved it extensively in *CH(3)1* homozygotes. Elevated sensitivity could also be induced by injecting *Parp*-specific RNAi (Fig. 5C).

[00141] Many additional nuclease sensitivity tests were carried out to develop a picture of which genomic sequences and regions become nuclease sensitive in the mutant. All the repetitive sequences tested were strongly affected (Fig. 5B, data not shown). These include the transposons GATE, gypsy, mdg1, hoppel, the S element, 297, Idefix, the rDNA-specific R1 element, and the Stellate repeats. In contrast, no changes in micrococcal nuclease sensitivity of the unique euchromatic genes *actin 5C* and *rp49* were observed (Fig 5D). The single-copy *Parp* gene resides within a region of highly repetitive sequences including many of the transposons shown to be affected in deficient animals. We tested three *Parp* exons, including exon 3 which lies adjacent to Pm1, and found that they became much more accessible to nuclease digestion in *CH(3)1* homozygotes (Fig. 5C). Taken together, these observations suggest that reducing PARP activity selectively alters the chromatin structure of heterochromatic and repetitive sequences but not of euchromatic, single-copy DNAs.

[00142] **Expression of PARP-e but not PARP-I rescues *CH(3)1* mutations**

[00143] Despite strongly reduced *Parp* expression in *CH(3)1* mutant animals and the correlation between the mutant phenotype and the effects of removing PARP activity using RNAi, we sought to verify that *CH(3)1* mutates *Parp* by rescue. Because of its size and unclonable structure, it is impractical to attempt rescuing *CH(3)1* using genomic *Parp* DNA. Consequently, we generated constructs that express Parp-I or Parp-e cDNAs under the control of a UAS promoter. Following transformation we studied the effects expressing these cDNAs throughout many tissues using the Armadillo-GAL4 driver. Ectopic expression of Parp-I cDNA, but not Parp-e cDNA, in wild type flies causes rough eyes and abdominal cuticle defects (data not

shown). Parp-I expressing animals arrest at the pupal stage if two doses of the driver are present. Thus, as in mammals, excess PARP-I levels cause deleterious effects.

[00144] Expressing Parp-e cDNA in a *CH(3)1* mutant background revealed that the PARP-e isoform can completely suppress larval lethality and give rise to a small number of viable, fertile adults. Mutant flies bearing two copies of the Parp-e cDNA and driver can be readily maintained as a homozygous stock. In contrast, mutant animals expressing Parp-I cDNA die as third instar larvae but still develop significantly farther than in the absence of the construct. Nonetheless, the ability of *Parp* cDNAs to partially or wholly rescue *CH(3)1* animals demonstrates that the *CH(3)1* lesion directly disrupts *Parp* gene expression. We have therefore renamed the *CH(3)1* and *CH(3)4* alleles as $Parp^{CH1}$ and $Parp^{CH4}$ respectively.

[00145] Expressing *Parp* transcripts dramatically restores the nuclear morphology and the *Parp* expression of the mutant larvae. Parp-I expression causes a nucleolus to form that can be visualized with the specific antibody AJ1 in many but not all nuclei (Fig. 6A). The mosaic nature of the response, which may result from cell-to cell variation in either the production or effects of ectopic PARP-I, is likely to explain the failure of this construct to rescue fully. All nuclei in the Parp-e expressing animals appear wild type in morphology. Surprisingly, larvae rescued by Parp-e contain higher than wild type levels of both the 2.6 kb Parp-e and the 3.2 kb Parp-I mRNA species (Fig 6B). Thus, the enzymatically inactive PARP-e isoform may rescue *CH(3)1* by inducing production of Parp-I mRNA. Consistent with this model, ADP-ribosyl transferase enzymatic activity is also restored, because the amount of poly(ADP-ribose)-containing protein detectable by anti-poly(ADP-ribose)-specific antibody increases to well above wild type levels (Fig. 6C, 117 kd PARP-I band). PARP-e may induce a more physiological pattern of Parp-I expression, leading to fewer deleterious effects than when Parp-I is mis-expressed globally. How expression of an enzymatically inactive protein rescues Parp-I expression and ADP-ribosyl transferase activity is discussed below.

[00146] **PARP and SIR2 modify GAL4/UAS variegation**

[00147] Since the *Parp* gene is located in heterochromatin and acts on the chromatin structure of repetitive DNA sequences, we investigated whether it functions as an enhancer or suppressor of variegated position effects. Neither $Parp^{CH1}$ nor $Parp^{CH4}$ altered the level of w^{m4} variegation, a standard test for modifiers of classical position-effect variegation (data not shown). However, we did notice that *Parp* strongly effects the variegated expression commonly exhibited by many UAS/GAL4 constructs (Brand and Perrimon, 1993). For example, in the presence of only one wild type dose of *Parp*+, the variegation of an epitope-tagged mitochondrial protein (Tim17B-DsRed) driven from a UAS promoter is strongly enhanced (Fig. 7A-B). At six different tested

sites of integration, DsRed expression is virtually silenced in a $Parp^{CH1}$/+ background, while at the seventh site, non-variegated expression becomes variegated. Altering the dose of *Sir2* was found to have the opposite effect and dominantly suppress UAS-Tim17B-DsRed variegation (Fig. 7C). Similar reciprocal effects were observed with two other tested constructs, UAS-Parp-I-DsRed (not shown) and UAS-Sir2-DsRed (Fig. 7D-F), suggesting that the dosage of *Parp* and *Sir2* may affect the expression of UAS/GAL4 constructs generally. PARP and SIR2 levels may alter the chromatin structure of sequences such as those in UAS and GAL4 constructs that are prone to silencing during development.

[00148] PARP regulates chromatin structure during development

[00149] PARP is a conserved protein known to play critical roles that help restore and maintain genomic integrity (reviewed by de Murica and Shall, 2000). By identifying lethal *Parp* mutations, we showed that *Drosophila Parp* also plays an essential role during the lifecycle in the absence of external stresses. Many genes have been identified previously that act in both DNA repair and during development (Baker *et al.* 1976. *Proc. Natl. Acad. Sci. USA.* **73:** 4140-4144; Gatti *et al.* 1989. *Genes Dev.* **3:** 438-53). However, the phenotype of *Parp* mutants differs from those of other genes in this class, which typically produce third instar larvae deficient in diploid tissue as a result of defects in the mitotic cell cycle.

[00150] Our experiments suggest that *Drosophila Parp* plays a special and fundamental role in organizing chromatin on a global scale. *Parp* mutant cells lack nucleoli and contain unusually nuclease-accessible repetitive sequences. Both the heavy expression of Parp-e and Parp-I in oocytes and early embryos and the early onset of these defects suggest that a major role for PARP occurs as development begins. At fertilization, the zygote genome is quiescent and unregionalized, but during the final cleavage divisions heterochromatin becomes distinguishable from euchromatin, nucleoli form, and specific gene transcription begins. Zygotic PARP activity may be needed to carry out these changes, which are reminiscent of the amphibian "mid-blastula transition." The strong enrichment of epitope-tagged PARP in nucleoli and on heterochromatin is consistent with such a role. When *Parp* function is limited by a declining maternal pool, chromatin may not regionalize normally, stunting further development.

[00151] Our observations argue that the role of *Parp* is not limited to the initial stages of development, however. Programmed changes in chromatin organization continue after blastoderm formation in concert with cell differentiation (reviewed in Hagstrom *et al.* 1997. *Curr. Opin. Genet. Dev.* **7:** 814-821). The effects of reducing *Parp* expression later in embryonic development using RNAi, and the influence of *Parp* dosage on GAL4/UAS variegation, indicate that it also participates in organizing chromatin domains during later

embryonic and larval growth. PARP plays a positive role in expressing euchromatic UAS constructs since reduced *Parp* dosage enhances the variegation of these transgenes. Furthermore, *Parp* function is likely to be specially required for larval metamorphosis, since up to 50% of mutant larvae were arrested at precisely this stage. Thus, PARP influences both the expression and silencing of particular euchromatic and heterochromatic sequences at diverse times during *Drosophila* development.

[00152] PARP may act by modifying chromosomal proteins

[00153] Enzymes that add or remove phosphoryl, acetyl or methyl gbe has been reported to associate with polynucleosomes *in vivo* (Leduc *et al.* 1986). We observed a strong reduction in the levels of protein ADP-ribosylation in PARP mutants. Many of the modified proteins detectable with antibodies that recognize protein-ADP(ribosyl) groups are located along chromosomes, and are particularly enriched in nucleoli and in the heterochromatic chromocenter, regions strongly affected by *Parp* mutations. These observations support the idea that PARP acts on *Drosophila* chromatin by ADP-ribosylating chromatin proteins.

[00154] PARP-e autoregulates the activity of a complex *Parp* gene located within heterochromatin

[00155] Our structural characterization of the *Parp* gene reveals that both the gene itself and its surrounding chromosomal region are complex. The *Parp* locus is localized in 3R heterochromatin near band h55, where it spans at least 150 kb. At least two promoters are utilized and the upstream promoter, Pm2, produces a transcript encoding a novel protein isoform, PARP-e, primarily during oogenesis and early larval development. Four other genes reside nearby and are transcribed in the same direction. In contrast, most of the DNA located outside gene exons consists of diverse transposable elements that are oriented opposite to the genes, perhaps as a result of selection to minimize the disruptive effects of transposon-encoded transcription and splicing signals. Much remains to be learned about the number, structure, regulation and evolution of heterochromatic genes (reviewed by Weiler *et al.* 1995. *Annu. Rev. Genet.* 29: 577-605; Cook *et al.* 1994. *Proc. Natl. Acad. Sci. USA.* 91: 5219-5221). The *Parp* region may now serve as a valuable model for detailed studies of these issues.

[00156] Our experiments suggest that *Parp* itself is subject to novel regulatory mechanisms. $Parp^{CH1}$ likely disrupts Parp-e transcription from Pm2, but homozygotes also have greatly reduced levels of Parp-I mRNA and of PARP activity, despite the fact that Pm1 is located at least 75kb downstream from the $Parp^{CH1}$ insertion site. Thus, Parp-e production appears to be essential for transcription of Parp-I from Pm1. It is difficult to rule out the existence of additional promoters or splice forms of PARP transcripts. However, the fact that expression of a

cDNA encoding PARP-e rescues lethality, Parp-I mRNA production and ADP ribosyl-transferase activity argues strongly that PARP-e autoregulates *Parp* transcription. Indeed, Parp-e expression may be rate-limiting for Parp-I transcription because overproduced Parp-e from the rescue construct was associated with elevated levels of Parp-I mRNA (Fig. 6C).

[00157] There are two basic ways in which Parp-e might control Parp-I transcription. PARP-e may simply function as a factor that activates transcription from Pm1. Alternatively, it may function by a novel mechanism related to its action on heterochromatin. The Pm1 promoter and surrounding sequences may need to acquire a compact, heterochromatic chromatin state for activity. Zygotic PARP-e produced near the onset of development would facilitate heterochromatin formation, thereby activating Pm1 and Parp-I production. Simultaneously, this chromatin transition might shut off or limit Parp-e production from Pm2. Such a feedback switch would link PARP production to the chromatin state and might represent a mechanism utilized by other heterochromatic genes. Two other such genes were shown recently to require the heterochromatin-specific HP-1 protein to be efficiently expressed (Lu *et al.* 2000. *Genetics.* 155: 699-708).

[00158] **PARP may remodel and maintain chromatin domains**

[00159] Previous studies of the role played by PARP during DNA repair have led to a model of how it acts on chromatin (reviewed in Zeigler *et al.* 2001). Following DNA damage, inactive PARP-I protein located near the damaged region binds to DNA breaks, activating the catalytic site, and begins to transfer ADP-ribose groups to the chromatin proteins located in the immediate vicinity and to the PARP automodification domain. The modified proteins are released from the DNA, allowing repair enzymes to access the damaged region. When repair is complete, the ADP-ribosyl groups are removed by a specific glycosylase and the disrupted chromatin reassembles. During this time, automodified PARP may serve as a local storage site for the dissociated chromatin proteins preventing them from diffusing away and mixing with general pools (Althaus, 1992). The local nature of the disruption may help to ensure that repair does not inadvertently lead to alterations in the pre-existing state of chromatin programming.

[00160] We propose that the role played by PARP in DNA repair, as described above, represents just one instance of a general function PARP carries out to re-program chromatin at multiple points during the life cycle. Inactive PARP molecules located in many chromosome regions may be subject to activation by particular developmental and environmental stimuli in addition to DNA damage. Following such stimulation, activated PARP would catalyze the dissociation of chromosomal proteins in the affected domain. Introducing new or differentially modified chromosomal proteins to the affected site in conjunction with PARP activation would cause the

local chromatin state to be specifically altered when ADP-ribosyl residues are subsequently cleaved and the dissociated proteins re-assembled. Such a mechanism would allow chromatin re-modeling to be precisely limited to particular chromosome regions by spatially controlling the sites of PARP activation and protein delivery. It might also explain many previous observations concerning the transcriptional role of PARP and its interaction with transcription factors.

[00161] Our results suggest that PARP acts to maintain certain chromatin domains as well as to remodel them. For example, copia sequences in animals that had already formed heterochromatin became nuclease sensitive when PARP levels were gradually reduced in developing embryos using RNAi. Even when enzymatically inactive, PARP molecules remain associated with many chromosome regions and may play essential structural roles. Disruption of these roles may be responsible for some of the effects caused by loss of the enzymatically inactive PARP-e isoform, and for some of the deleterious effects of PARP-I over-expression. Our findings emphasize the importance of learning more about the properties of PARP molecules within specific chromosome regions and how they change during chromatin re-programming. Finally, they suggest ways in which manipulating PARP molecules might allow chromatin re-programming to be experimentally controlled.

[00162] **PARP is distributed widely along chromosome but ADP-ribose modified proteins are enriched in polytene chromosome puffs**

[00163] We used flies bearing PARP-DsRed- or PARP-EGFP- transgenes (Tulin *et al.* 2002) to further investigate the distribution of PARP protein on chromosomes (Fig. 8). PARP associates with the chromatin of diploid cells although at lower levels than in nucleoli (Fig. 8A). The incorporation pattern of biotinylated-NAD into protein could be visualized in polytene larval cells, and was very similar to the pattern of PARP-DsRed (Fig. 8D, 8E). This suggests that PARP molecules with a low level of enzymatic activity coat chromosomes. In contrast, the pattern of staining with an antibody that specifically recognizes large clusters of protein-bound NAD-ribose moieties is discontinuous and strongly enriched at a limited number of euchromatic sites (Fig. 8F). When chromosomes were squashed under appropriate conditions (Examples), many of these sites could be seen to correspond to polytene chromosome puffs (Fig. 8G).

[00164] Interestingly, we observed that high levels of modified proteins were not observed prior to puffing (Figs. 8H, 8I) and that the actual level of PARP protein in puffs was similar to other regions (Fig. 8J). These observations suggested that PARP protein becomes strongly activated within puffs and modifies local proteins by adding ADP-ribose moieties. In the course of these studies, we noticed that while the average level of PARP expression is greatly reduced in the

Parp^{CHI} mutant animals (Tulin *et al.* 2002) a low level of variegated expression continues in this P-element induced mutation, as scattered nuclei still contained nucleoli (Fig. 8C, arrow).

[00165] Heat shock induced expression of hsp70 is greatly reduced in PARP- larvae

[00166] A short heat shock strongly induces puffing at a small number of specific loci containing stress response genes (reviewed in Lindquist S. 1986. Ann. Rev. Biochem. 55:1151-91) including the 87A and 87C puffs containing clustered genes encoding the Hsp70 chaperone (Fig. 9A, 9B). Prior to heat shock, only normal background amounts of poly(ADP-ribose)-modified proteins are present at the 87A and 87C loci (Fig. 9C, 0 minutes). Within just 10 minutes after shifting to 37 °C, ADP-ribose modified proteins accumulate throughout the newly forming puffs (Fig. 2C, 10 minutes). The amount of these modified proteins begins to fall sometime thereafter, and the puff itself will soon regress (Ashburner. 1970. Adv. Ins. Phys. 7, 1-95). These observations raise the question of whether the increase in protein ADP-ribosylation is a cause or effect of the process of heat shock induced puffing.

[00167] To determine if the *Parp* gene encodes the enzyme responsible for this increase, and if it is required for puffing, we wanted to determine if heat shock induced puffs formed normally in Parp mutant larvae. Unfortunately, *Parp*-defective animals die as second instar larvae, before salivary gland polytene chromosomes grow large enough to recognize banding patterns or specific puffs. However, the small puffs that form in wild type l2 chromosomes can be visualized using anti-RNA polymerase antibodies, which recognize the large accumulation of this enzyme at puff loci (Fig. 9F, G). Moreover, it was possible to recognize the 87A 87C "double" puff specifically. These studies showed that the average size of the 87A and 87C puffs was reduced at least 3-fold in *Parp*^{CHI} larvae. We suspected those cells within *Parp*^{CHI} larvae that still were able to form small heat shock puffs, might retain a small amount of maternal or leaky *Parp* expression. Consequently, we double stained for puffs and for AJ1 nucleolar antigen. Cells that formed substantial puffs (3.0-4.0) still had nucleoli, whereas cells with very small puffs showed only cytoplasmic staining (data not shown). We concluded that PARP was required to form normal heat shock puffs, most likely by modifying proteins at the puff site. A similar blockage of puff formation was observed in third instar larvae within 30 minutes of injecting the specific PARP inhibitor, 3-aminobenzamide.

[00168] If the chromatin alterations that give rise to a puff are important for gene activity, then Hsp70 production should be reduced following heat shock in *Parp*^{CH} compared to wild type larvae. Western blots showed that the amount of Hsp70 protein recognized by specific antibodies was reduced 5-10-fold in the mutant. Thus, Parp is needed to form normal heat shock puffs and to express normal levels of puff encoded proteins.

[00169] PARP is required for anti-bacterial immunity

[00170] Heat shock genes all utilize a common transcription factor called HSF (Wu, *Methods Enzymol.* 170: 269 (1989)). Mice deficient in Parp1 display immune defects and are unable to normally induce immune responses that require the NF-κB family of transcription factors (see deMurica *et al.* 2000). A major mechanism of resistance to extracellular microbes in insects such as Drosophila is provided by innate immunity genes (reviewed by Hoffman *et al.* 2002. *Nat Immunol.* 3: 121-6). These genes encode antimicrobial peptides and their rapid induction following infection is controlled by NFκB-related transcription factors (Han *et al.* 1999. *J Biol Chem.* 274: 21355-61).

[00171] Consequently, to look for other gene families that might require PARP, we tested the resistance of wild type and $Parp^{CH1}$ larvae to the injection of approximately $2x10^4$ *E.coli* bacteria injected into their haemolymph. More than 95% of wild type but less than 7% of $Parp^{CH1}$ larvae survive such a challenge. Even normal, unchallenged mutant animals frequently acquired spontaneous intracellular bacterial infections (Fig. 10A). In tissues showing variegated *Parp* activity we noticed that bacteria were found preferentially in cells that lacked *Parp* activity (Fig. 10B).

[00172] We compared the ability of wild type animals to induce two innate immunity genes, Diptericine and Drosomycin, using Diptericine-lacZ and Drosomycin-GFP reporter genes that can be recognized by specific anti-sera (Fig. 10D). Both genes were strongly induced in wild types following bacterial challenge, but under the same conditions their levels increased little if at all in $Parp^{CH1}$animals. To determine if Parp might be required at the level of chromatin, we injected bacteria along with biotinylated-NAD into l3 larvae and analyzed polytene chromsomes using avidin staining three hours later (Fig. 10C). Evidence that NAD was heavily incorporated at one or more specific loci was observed, suggesting that some loci do form puffs in response to bacterial infection.

[00173] PARP may facilitate diverse types of chromatin remodeling

[00174] Previously, we reported that PARP is required to form and/or maintain normal heterochromatin, to repress copia retrotransposon transcription, and to form and/or maintain nucleoli (Tulin *et al.* 2002). The experiments reported here extend the chromosomal processes that require *Parp* function to include the induction of specific genes- those encoding Drosophila heat shock proteins, and at least some innate immunity genes. PARP's proposed mechanism of action during DNA repair suggests a model (Fig. 11) that can unify many or all of these functions. Canonical PARP proteins such as mouse PARP1 are thought to act as a reversible chromatin removal device at sites of DNA damage. First, PARP senses lesions and activates its

catalytic domain, causing it to modify nucleosomal histones, transcription factors and other chromosomal proteins so that they dissociate from DNA. At the same time, activated PARP turns off its own catalytic activity by automodification. While present, the ADP-ribosyl chains may bind the removed chromatin proteins and tether them near their original location. Following repair of the lesions and removal of poly(ADP)-ribose groups by glycosylase, the chromatin proteins are freed and reassemble onto the DNA.

[00175] For a similar model to explain developmentally or environmentally controlled chromatin re-modeling, this general sequence of events must change in two ways. First, signals other than DNA lesions must be able to activate PARP. PARP interacts specifically with many other proteins, some of which may activate the COOH terminal catalytic domain. PARP contains potential target sites for casein kinase 2, and may itself be subject to covalent modifications that might facilitate activation. Whatever the mechanism, it would be important that it take place only with appropriate chromosome domains and at the appropriate time. This suggests that specific transcription factors with which PARP interacts are strong candidates as co-activators of PARP activity.

[00176] The second requirement is that new co-factors or modifications occur while the chromatin proteins have been removed so that they reassemble into a different state than before. Many sites of PARP activation, such as nucleoli and puff loci, may reassemble in their original state, like sites following DNA repair. However, in certain cases, as during heterochromatin formation, or some hormonally induced puffs, a new chromatin state may arise. For this end, new chromatin proteins may be added, and existing proteins may be modified while dissociated and bound to ADP-ribose polymers. The disassembly of protein complexes is likely to facilitate re-assembly into new patterns. The same environmental or developmental signals that activate PARP might induce the relevant new proteins and modifying activities to program the outcome.

[00177] **Puffs may be a visual manifestation of PARP-mediated chromatin remodeling**

[00178] The direct visualization of the chromatin changes during puffing is one of the strongest arguments for the model of Fig. 11. However, the mechanism and function of these dramatic chromatin alterations have remained enigmatic. While the promoter strength and length of the underlying transcription unit can affect the size of puffs induced by heat shock transgenes (Simon *et al.* 1985. *Cell.* 40: 805-17), puffing and transcription are separable (Simon *et al.* 1985. *Chromosoma* 93:26-30); Meyerowitz *et al.* 1988). The chromatin surrounding the hsp70 genes is profoundly altered by puffing. Nuclosomes lose their regular association with DNA (Cartwright *et al.* 1986. *Mol. Cell. Biol.* 6, 779–791) and the DNA becomes as extended as naked DNA (Simon *et al.*, 1985). Our experiments provide a plausible mechanism for these

extensive changes. The question of why genes such as hsp70 and ecdysone response genes, but not many other highly transcribed genes, undergo these major chromatin changes upon activation remains less clear. Loosening and removal of nucleosomes may allow an increased polymerase elongation rate and hence higher rates of protein production. If so, then puff loci must have evolved transcriptional mechanisms that are resistant to disruption by PARP activation. During repair Poly(ADP-ribose) addition is thought to inhibit transcription. Poly(ADP-ribose) addition to TATA binding protein (TBP) and to transcription factors such as YY1, p53 (Mendoza-Alvarez *et al.* 2001) and CREB blocks their ability to bind DNA *in vitro*. However, TBP and transcription factors that are already bound to DNA resist modification and are not released (Oei *et al.* 1998). The preformed transcription complexes found on heat shock genes (reviewed in Lis *et al.* 1995. In: Elgin, S.C.R. (Ed.), Chromatin Structure and Gene Expression. IRL Press, Oxford, pp. 71–88) might therefore provide an initial resistance to inhibition. Large amounts of poly(ADP-ribose) polymer might have functions completely separate from transcriptional activation, such as providing temporary storage sites for chaperone complexes containing nuclear proteins. Regardless of the exact mechanisms, our findings indicate that PARP activation does not inevitably lead to the dissociation of all chromatin proteins, and that high levels of PARP activation are compatible with some ongoing chromosomal functions.

[00179] Parp function is likely to be required to form puffs generally, and not just for heat shock puffs we studied. All the puffs we observed had high levels of ADP-ribose modified proteins, including the ecdysone induced puffs such as 74A and 75B (Fig. 9). We previously reported that $Parp^{CH1}$ mutant larvae frequently arrest development precisely at the moult between the second (l2) and third (l3) larval instars (Tulin *et al.* 2002). Such molts are induced by a rise in the titer of juvenile hormone, and induce a series ecdysone response genes (Thummel 2000). Our results suggest that the developmental arrest of *Parp* larvae results from a failure to express ecdysone response genes.

[00180] **Parp may act by other mechanisms that do not involve chromatin removal**

[00181] In some instances, PARP has been proposed to act directly as a transcription factor or chromatin modulator. In mammals, NF-κB and PARP-1 form dimers (Hassa *et al.* 1999; Chang *et al.* 2001. *J Biol Chem.* 276: 47664-70). These complexes may activate NF-κB dependent gene transcription, a function requiring neither PARP DNA binding nor catalytic activity (Hassa *et al.* 2001. *J Biol Chem.* 276: 45588-97). Alternatively, the complex may be inactive, but PARP automodification would disrupt its association with NF-κB, releasing NF-κB for activation (Chang *et al.* 2001). The nature of the PARP1 requirement *in vivo* has not been resolved,

however. *Parp*-deficient Drosophila were found to have also have defects in immune function and in NF-κB-dependent gene transcription. Further study immune gene induction in normal and mutant flies is likely to reveal if *Parp* is required at the level of transcriptional initiation or chromatin structure.

[00182] PARP forms stable protein-protein complexes with numerous other chromosomal proteins. These interactions might in some cases modulate chromatin structure by mechanisms that do not require catalytic activity. For example, PARP associates with the mammalian YY1 transcription factor. In Drosophila, the YY1 homologue Pleiohomeotic is likely determinant for repression complexes mediated by the Polycomb protein.

[00183] There are additional reasons to believe that not all actions mediated by PARP take place via chromatin changes. Both mammalian cells and Drosophila produce multiple PARP isoforms as well as related proteins sharing some protein domains. A number of these proteins contain the PARP catalytic domain consensus without the DNA binding or automodification domains. In addition, molecules such as PARP-e (Tulin *et al.* 2002), lack the catalytic domain, and PARP-e is required for the production of all *Parp* isoforms. These PARP-e related molecules may act directly as transcription factors or chromatin proteins. Thus, PARP-e related proteins are abundant and versatile proteins that likely play diverse roles.

[00184] **Use of Parp for gene re-programming**

[00185] These studies suggest that PARP could be used to experimentally reprogram chromatin, a capability that would have many useful applications. For example, a specific gene could be activated in a differentiated cell type where it is normally inactive and in a suppressed chromatin state. First, endogenous chromosomal PARP molecules would be activated at the site of such a gene by engineering local DNA damage, or through the binding of PARP-activating factors that act at normal puff sites. The activated PARP molecules will then modify local chromatin proteins, including histones, causing some to dissociate and the chromatin structure to loosen. If the transcription factors needed for gene transcription are present or are simultaneously provided, the desired gene should begin to be transcribed. In some cases this may be sufficient for the desired effect, however, it would likely be possible by further actions to make the reprogrammed gene remain active. If appropriate chromatin and chromatin-modifying proteins are expressed prior to the downregulation of PARP activity, the chromatin surrounding the gene is likely to re-assemble in an active state. A better understanding of the role of PARP in normal chromatin re-programming will likely facilitate the development of such methods.

[00186] Having now fully described this invention, it will be understood by those of ordinary skill in the art that the same can be performed within a wide and equivalent range of conditions,

formulations and other parameters without affecting the scope of the invention or any embodiment thereof. All patents and publications cited herein are fully incorporated by reference herein in their entirety.